48

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)
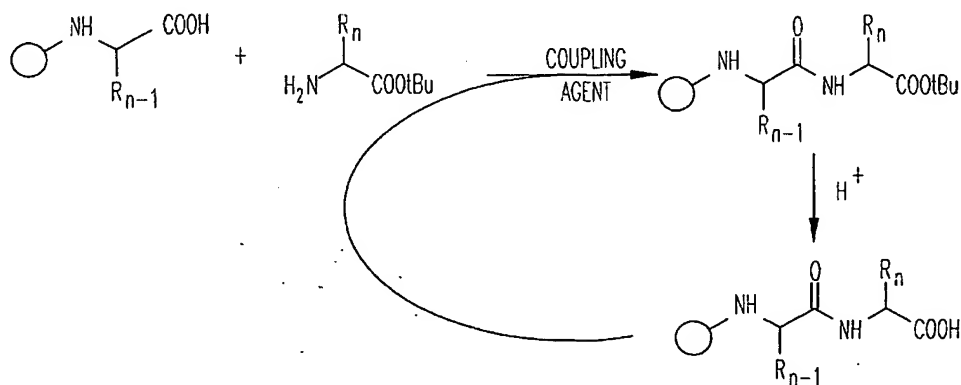
(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **GUTHEIL, William, G.** [US/US]; 4906 Holly Street, Kansas City, KS 64112 (US). **XU, Qingchai** [CN/US]; 1311 Volker Boulevard, Apt. 2E, Kansas City, MO 64110 (US).

(74) Agents: STEFFEY, Charles, E. et al.; Schwegman, Lundberg, Woessner & Kluth, P.O. Box 2938, Minneapolis, MN 55402 (US).

(54) Title: INVERSE SOLID PHASE SYNTHESIS OF PEPTIDES

(57) Abstract: The present invention provides a process for preparing a peptide of formula (I): Sub-[L]-[NH-A-C(O)]$_{n+m}$-OH comprising: (a) reacting an immobilized compound of formula (II): Sub-[L]-[NH-A-C(O)]$_n$-OH with an amino acid ester or peptide derivative of formula (III): H-[NH-A-C(O)]$_m$-O(tBu) in the presence of a coupling agent to yield a peptide compound of general formula (IV): Sub-[L]-[NH-A-C(O)]$_{n+m}$-O(tBu) (b) removing the tBu (t-butyl) group to produce a solid-support bound carboxylic acid or peptide derivative of general formula (I); wherein n is a positive integer.

WO 02/094857 A1

INVERSE SOLID PHASE SYNTHESIS OF PEPTIDES

Background of the Invention

The standard methods for the solid phase synthesis of peptides (SPPS) on beaded resins in the normal C-to-N direction are well developed, as they must be if long peptides are to be synthesized (reviewed in M. Bodanszky et al., "Peptide

10 Chemistry: A Practical Textbook," Springer-Verlag, NY (2d ed., 1993) and M. Bodanszky et al., "The Practice of Peptide Synthesis," Springer-Verlag, NY (2d ed., 1993)). These methods are based on attaching the carboxy terminus of an amino-protected amino acid to the resin. It essentially allows peptides to be synthesized without racemization when amino acids with N^{α}-protecting groups

15 of the urethane (carbamate) type were coupled in stepwise fashion. The amino-protecting group is removed, and the next amino acid with a free carboxy group and protected amino group is then added using standard coupling conditions. Standard peptide chemistry has been used to prepare solid phase peptide libraries of tremendous diversity (H. M. Geysen et al., Molec. Immunol., 23, 709 (1986);

20 R. A. Houghten et al., Nature, 354, 84 (1991); K. S. Lam et al., Nature, 354, 84 (1991)).

Peptide mimetic libraries (mimetic is used to indicate a modified peptide), based on the normal C-to-N direction of peptide synthesis, have been described. A peptide phosphinate library has been synthesized and used to find

25 potent and selective inhibitors of zinc metalloproteases (J. Jiracek et al., J. Biol. Chem., 270, 21701 (1995); J. Jiracek et al., J. Biol. Chem., 271, 19606 (1996); V. Dive et al., PNAS USA, 96, 4330 (1999)). A (hydroxyethyl)amine library has also been synthesized and used to find inhibitors of the prototypical aspartyl protease Cathepsin D (E. R. Kick et al., Chem. Biol., 4, 297 (1997)).

1

However, combinatorial libraries of polypeptides representative of serine and cysteine proteases have not been synthesized. This can be attributed to the fact that virtually all the effective classes of serine and cysteine classes, such as peptide aldehydes (R. C. Thompson, Biochem., 12, 47 (1973)), chloromethyl
5   ketones (CMKs) (G. Schoellmann et al., Biochem., 2, 252 (1963)), fluoromethyl ketones (FMKs) (M. H. Gelb et al., Biochem., 24, 1813 (1985); B. Imperiali et al., Biochem., 25, 3760 (1986)), and boronic acids (C. A. Kettner et al., J. Biol. Chem., 259, 15106 (1984); C. A. Kettner et al., J. Biol. Chem., 265, 18289 (1990)), are prepared by elaboration of a peptide at its C terminus. Thus, a need
10   exists for the development of combinatorial peptide libraries representative of the serine and cysteine protease classes. Such libraries would greatly improve the ability to develop potent and specific inhibitors of these enzymes. This, in turn, requires a free C terminus of an immobilized oligopeptide, which, in turn, requires the ability to synthesize peptides in the N-to-C direction: or "reverse
15   peptide synthesis."

Considering the importance of such C-terminally modified peptides and peptide libraries, relatively few reports have been devoted to the development of a direct method for peptide assembly in the inverse direction. See, for example, R. J. Broadbridge et al., Chem. Commun., 1449 (1998), K. H. Bleicher et al.,
20   Tett. Lett., 39, 4591 (1998); F. Bordusa et al., Angew Chim Int. Ed. Eng., 36, 1099 (1997); R. Leger et al., Tet. Lett., 39, 4147 (1998); R. Letsinger et al., J. Amer. Chem. Soc., 85, 5163 (1969); and 85, 3015 (1963).

Merrifield et al., J. Amer. Chem. Soc., 92, 1385 (1970), used protected amino acid hydrazides as building blocks for the C-terminal elongation of
25   peptides, followed by deprotection and subsequent reaction of the hydrazide function with nitrite allowed the next building block to be coupled by the azide method. However, the procedure is elaborate, requiring activation and coupling at low temperature with moderate yields.

Later investigations, mainly by Bayer and co-workers, have included the
30   use of base labile amino acid 9-fluorenylmethyl esters, which were coupled with N-hydroxybenzotriazole (HOBt)/diisopropylcarbodiimide (DIC) or N-[(1H-

2

benzotriazole-1-yl)-(di-methylamino)methylene]-*N*-methylmethanaminium
tetrafluoroborate *N*-oxide (TBTU)/*N*-methylmorpholine (NMM). B. Henkel et
al., Liebigs Ann./Recieul, 2161 (1997). In both cases, a 30-min. preactivation
step with a large excess of activator (8 equiv.) was included before addition of

5   the amino acid ester. Using TBTU/NMM extensively racemized products were
obtained, whereas coupling using HOBt/DIC gave moderate racemization but led
to significant formation of other byproducts.

        Sharma et al. have described a few C-terminally modified tetrapeptide
HIV-1 protease inhibitors, generated in the inverse direction. For example, see,

10   R. P. Sharma et al., published PCT applications WO 93/05065 (18 Mar. 1993)
and WO 90/05738 (31 May 1990) and Chem. Commun., 1449 (1998). Sharma's
approach relies on coupling of amino acid tri-*tert*-butoxysilyl (Sil) esters. More
recently, A. Johannsson et al., J. Comb. Chem., 2, 496 (2000) described a
modification of the method of Sharma et al. that involves the coupling of a

15   photolabile resin-bound C-terminal amino acid with excess amounts of amino
acid tri-*tert*-butoxysilyl (Sil) esters, using HATU as coupling reagent and 2,4,6-
trimethylpyridine (TMP, collidine) as a base. Levels of epimerization were
considerably lower than those reported for other N-to-C methods, usually ca. 5%
and occasionally even below 1%. However, in unfavorable cases, as in the

20   coupling of Ile to Ser, the level was higher (ca. 20%). Also, this method requires
the synthesis of sensitive zwitterionic tris (t-butoxy silylesters) and familiarity
with photolytic techniques.

        Thus, a continuing need exists for simple and efficient methods for the
reverse synthesis of peptides and peptide mimetics, particularly for the synthesis

25   of oligopeptide mimetic libraries useful for high-throughput drug screening.

<div align="center">

Summary of the Invention

</div>

        The present invention provides a process for preparing a peptide of
formula (I):

30   Sub-[L]-[NH-A-C(O)]$_{n+m}$-OH   (I)

      comprising:

<div align="center">

3

</div>

(a)   reacting an immobilized compound of formula (II):

$$\text{Sub-(L)-[NH-A-C(O)]}_n\text{-OH} \qquad \text{(II)}$$
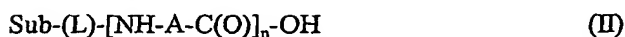
with an amino acid ester or peptide derivative of formula (III):

$$\text{H-[NH-A-C(O)]}_m\text{-O(tBu)} \qquad \text{(III)}$$

5   in the presence of a coupling agent to yield a peptide compound
        of general formula (IV):

$$\text{Sub-[L]-[NH-A-C(O)]}_{n+m}\text{-O(tBu)} \qquad \text{(IV)};$$

(b)   removing the tBu (t-butyl) group to produce a solid-support
        bound carboxylic acid or peptide derivative of general formula

10   (I);

wherein n is a positive integer, e.g., 1-10, preferably 1-5; m is a positive integer,
e.g., 1-10, preferably 1-5. Sub is a solid support such as a polymer or glass
substrate, preferably comprising free OH, or NH_2 groups. L is a linker to the
amino acid ester or peptide derivative. Each A independently is the residue of a

15 naturally occurring or synthetic amino acid; a peptide residue, such as an
oligopeptide or polypeptide; or the structure -NH-A represents a heterocyclic
group, e.g., a C_5-C_{10} heterocyclic group comprising 1-2 O, N and/or S such as
pyrrolidine, indole or imidazole. This approach is summarized in Fig. 1, wherein
CHR represents the residue of an α-amino acid and O represents Sub.

20   The present method optionally comprises, prior to step (a): forming a
solid-support bound compound of general formula (VI):

$$\text{Sub-[L]-[NH-A-C(O)]}_n\text{-O(tBu)} \qquad \text{(VI)};$$

by reacting a functionalized solid support of general formula Sub[L]X
with a compound of formula: $\text{H-[NH-A-C(O)]}_n\text{-OH}$, wherein X is a group such

25 as CO_2H, or an activated carboxylic acid ester, that links L to NH_2-A by
elimination of XH; and removing the t-Bu group to yield a compound of formula
(II). Preferred moieties [L]X include $-OC(O)(CH_2)_2CH(NHZ)CO_2H$,
$HNC(O)(CH_2)_2CH(NHZ)CO_2H$, and $-C(O)(CH_2)_2CO_2H$, wherein Z is H or an
amino group protecting group such as benzyloxycarbonyl (Cbz).

30   The present method preferably further comprises the step of cleaving
compound (IV) at the L-NH bond to yield a compound of formula (IX):

$$H\text{-}[HN\text{-}A\text{-}C(O)]_{n+m}\text{-}OH. \qquad\qquad (VIII)$$

or of cleaving compound (IV) at the Sub-(L) bond to yield a compound of
formula (X):

$$H\text{-}(L)\text{-}[NH\text{-}A\text{-}C(O)]_n\text{-}O(tBu) \qquad\qquad (X),$$

5   wherein the amino group protecting groups are then removed, as necessary.

The present method preferably further comprises, following step (b);
carrying out, x times, the steps of reacting compound (I) with a compound of
formula (III) and removing the tBu group to yield a compound of formula (VIII):

$$Sub\text{-}(L)\text{-}[NH\text{-}A\text{-}C(O)]_{n+m(x+1)}\text{-}OH \qquad\qquad (VIII).$$
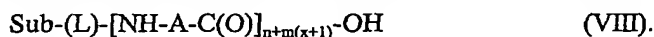
10

For example, if n and m are both 1, and A is an amino acid residue,
carrying out the steps 3 times will yield an immobilized oligopeptide having 5
peptidyl residues, not including L. The values $n+m(x+1)$ can be any integer up
to the maximum number of residues yielding a useful polypeptide or polypeptide

15   analog, e.g., about 75-100. Compound (VIII) can also be cleaved at the sub-(L)
bond or the (L)-NH bond to yield compounds analogous to compounds (IX) and
(X) above.

Novel compounds, particularly immobilized peptides, are also within the
scope of the present invention, e.g., compounds (I), (II), (III), (IV), (VI), (VIII)

20   and the like.

Preferably, each A is individually the residue of an α-amino acid, most
preferably, a residue of a naturally occurring L-amino acid, such as the
alkylidenyl or substituted alkylidenyl residues derived from glycine (Gly) ($-CH_2-$
), alanine (Ala) ($CH_3\overset{|}{C}H-$), serine (Ser) ($CH_2(OH)\overset{|}{C}H-$), threonine (Thr)

25   ($CH_3CHOH\overset{|}{C}H-$), valine (Val) ($CH_3CH(CH_3)\overset{|}{C}H-$), leucine (Leu) (-
$CH_3CH(CH_3)CH_2\overset{|}{C}H$), isoleucine (Ile) ($CH_3CH_2CH(CH_3)\overset{|}{C}H-$), cysteine (CySH)
($CH_2(SH)\overset{|}{C}H-$), cystine (CyS-SCy) $[-SCH_2\overset{|}{C}H-]_2$, phenylalanine (Phe)
($PhCH_2\overset{|}{C}H-$), tyrosine (Tyr) ($4\text{-}HOPhCH_2\overset{|}{C}H-$), proline (Pro) (pyrrolidin-2-yl),
hydroxyproline (4-hydroxy-2-pyrrolidinyl), tryptophan (Trp) ((indol-3-

30   yl)$CH_2\overset{|}{C}H-$), aspartic acid (Asp) ($HOOCCH_2\overset{|}{C}H-$), glutamic acid
($HOOCCH_2CH_2\overset{|}{C}H-$); histidine (His) ((imidazol-3-yl)$CH_2\overset{|}{C}H-$), lysine (Lys)

$(H_2N-(CH_2)_4\overset{|}{C}H-)$, or arginine (Arg) $(H_2NC(=NH)(CH_2)_3\overset{|}{C}H-)$. Free CO_2H,
NH_2, OH, or SH groups on A groups can optionally be protected with suitable
protecting groups (Z) such as tBoc, Cbz, acyl, benzyl, silyl, hemiacetals, or alkyl.

The present method can also be readily adapted to form C-terminal
modified peptides, for example, by reacting compound (I) or (VIII) with
$H_2N(alkyl)CH(OH)CF_3$ to yield the corresponding amide of (I) or (VIII) and
oxidizing the CH(OH) moiety to yield a trifluoromethyl ester of general formula:

$$Sub-(L)-[NH-B-C(O)]_q-NH(alkyl)C(O)CF_3,$$
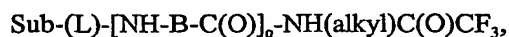
wherein q is n+m or n+m(x+1), respectively, followed by cleaving the compound
from Sub to yield a compound of formula:

$$H-L-[NH-A-C(O)]_q-NH(alkyl)C(O)CF_3.$$
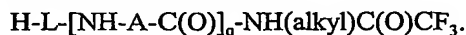
Compound (I) or (VIII) can also be reacted with $H_2N(alkyl)OH$ to yield
the hydroxyalkylamide of (I) or (VIII) and oxidizing the terminal hydroxy group
to yield a terminal aldehyde of formula:

$$Sub-(L)-[NH-A-C(O)]_q-NH(alkyl)CHO.$$
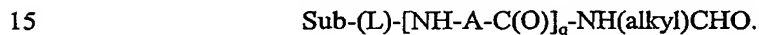
The terminal CHO group can be protected by conversion into an ethylene
glycol acetal, a propylene glycol acetal, an ethylene dithiol acetal or a 1,3-
dithiane, and cleaved from the substrate

$$H-(L)-[NH-A-C(O)]_q-NH(alkyl)CH \overset{\displaystyle X}{\underset{\displaystyle X}{<}} \left. \right] (CH_2)_p$$
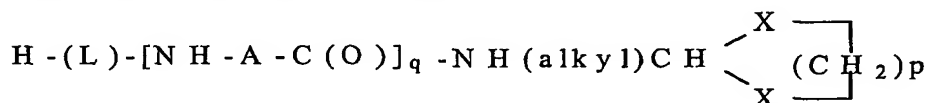
wherein X is O or S, and p is 0 or 1. The group (alkyl) is preferably a (C_1-C_6)
alkyl moiety, including the alkylidenyl or substituted alkylidenyl moieties
described hereinabove.

Compound (I) or (VIII) can also be reacted with a boroamine to yield a
compound of formula:

$$Sub-[L]-[NH-A-C(O)]_q-NH(A)B(OR)_2$$
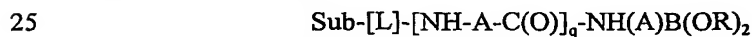
wherein R is (C_1-C_6)alkyl, phenyl or the residue of an organic diol such as
pinanediol, catechol, pinacol, diethanolamine and the like. See, Kettner (U.S.

6

Pat. No. 4,499,082). R can be cleaved from Sub to yield a compound of the formula:

$$H-[L]-[NH-A-C(O)]_q-NH(A)B(OH)_2.$$

As noted above, in some cases, it is desirable to cleave the [L]-NH bond

5   of Sub-[L]-NH to free the peptide from Sub.

The corresponding terminal chloromethyl ketone, $C(O)CH_2Cl$, can be prepared from the free CO_2H compounds as disclosed below.

In accord with the present method, reversing the direction of peptide synthesis provides the chemically versatile carboxy group for modification. This

10   approach has significant advantages for preparing solid phase peptides with either a free carboxy terminus or a carboxy terminal peptide mimetic. These advantages include:

1)   The ability to prepare immobilized peptides with a free carboxy terminus;

2)   The ability to prepare immobilized peptides with a directly modified

15   carboxy terminus, for example, peptide esters, peptide aldehydes, and peptide chloromethyl ketones; and

3)   The ability to introduce other monomer groups, such as amino acid mimetics such as boronic acids and trifluoromethyl ketones, onto the carboxy terminus.

20   Such derivatives, such as peptide aldehydes and chloromethyl ketones, can themselves serve as intermediates during the syntheses of other derivatives. For example, peptide chloromethyl ketones can be treated with nucleophilic reagents, which will displace the chloride ion, to make further derivatives. The same applies to peptide aldehydes. This synthetic versatility of the carboxy

25   group allows tremendous chemical diversity to be accessed via the inverse N-to-C peptide synthesis method described here.

As prepared in accord with the present method, peptide and peptide mimetic libraries can be screened for drug leads for nearly any desired target. The present invention can provide a novel set of peptide mimetic libraries for use

30   in such screens. Most notably, this invention provides a way to generate chemical substances of particular interest for the discovery of potent and specific

7

protease inhibitors, especially the serine and cysteine proteases. Most protease inhibitor classes are based on carboxy group chemistry. Examples include peptide boronic acids, peptide aldehydes, peptide chloromethyl ketones, and peptide fluoromethyl ketones. Proteases dependent diseases which can be

5   targeted using such compounds include hepatitis, AIDS, Alzheimer's disease, malaria and cancer, among others. The penicillin-binding proteins (PBPs) are also excellent targets for this synthetic method since they require a free carboxy terminus. New PBP inhibitors also are candidates as new antibacterial agents.

10   Detailed Description of the Invention

The present method provides a simple and effective method for synthesizing peptides in the N-to-C direction, based on amino acid t-butyl esters, as shown in Fig. 1. This method takes advantage of the availability of a number of amino acid t-butyl esters, and a list of commercially available amino acid t-

15   butyl esters appropriate for reverse peptide synthesis is given in Table 1. This list provides suitable derivatives to prepare L peptides containing all the standard L amino acids except Asp, Cys, Ser, and thr, as well as a number of non-standard and D amino acids.

Table 1: Commercially available amino acid t-butyl esters available

from Bachem appropriate for N-to-C peptide synthesis.

Ala-OtBu	Gln-OtBu	D-Leu-OtBu	D-Pro-OtBu
D-Ala-OtBu	Glu(OMe)-OtBu	Lys(Z)-OtBu	Sar-OtBu
β-Ala-OtBu	Glu(OtBu)NH$_2$	Met-OtBu	Thr-OtBu
Arg(Mtr)-OtBu	Gly(OtBu)-OMe	Orn(Z)-OtBu	Trp-OtBu
Asn-OtBu	Gly-OtBu	Phe-OtBu	Tyr-OtBu
Asp(OtBu)-NH$_2$	His(1-Trt)-OtBu	Phg-OtBu	D-Tyr-OtBu
Asp(OtBu)-OMe	Ile-OtBu	Pro-OtBu	Val-OtBu
	Leu-OtBu		D-Val-OtBu

All amino acids are L unless otherwise indicated. Abbreviations: Standard
amino acids have their normal abbreviations. Other Abbreviations: Mtr = 4-
methoxy-2,36-trimethylbenzenesulfonyl, Orn = ornithine (2,5-diaminopentanoic
acid), OtBu = O-t-butyl, Phg = L-phenylglycine, Sar = sarcosine (N-methyl
glycine), Trt = trityl.

A number of conditions are expected to affect both coupling efficiency
and quality (racemization) of the final product. Syntheses can be monitored for
overall yield by HPLC and for racemization using Marfey's reagent (Marfey,
Calsberg Res. Commun., 49, 591 (1984); Adamson et al., Anal. Biochem., 202,
202 (1992)). Optimization can be performed for the coupling of Pro to Suc-Phe-
Leu, since addition of Pro should be more difficult than most amino acids, and
coupling to a dipeptide (Suc is the linker) allows racemization both at the
activated residue (Leu in this case) (Bodanski cited above (1993)). Phe can
provide a convenient chromophore for detection of products.

In normal (C-to-N) peptide synthesis, an excess of the activated carboxyl
component is used to drive the reaction to completion. In N-to-C synthesis, the
carboxyl group is anchored and cannot be generated in excess. This problem can
be solved by using carbodiimides, or other coupling reagents, which can be used
in the presence of the carboxyl component and an excess of the amine
component. A second difficulty concerns which protection chemistry to use for
the carboxyl group of the amine component. One recent report (Johansson et al.,

cited above (2000)) describes the use of amine components with a silyl-protected carboxyl group, the use of a photolabile attachment to the solid support, and the use of HATU, (O-(7-Azabenzotriazol-1-yl)-N, N, N',N'-tetramethyluronium hexafluorophosphate; or TATU (O-Benzotriazol-1-yl-N,N-N',N'-

5   tetramethyluronium tetrafluoroborate) as coupling agents. HBTU, and PyBOP can also be used as coupling agents.

These reagents can be used in about 2, 4, 6, and 8-fold molar excess. Bases 2,4,6-trimethylpyridine (TMP), diisopropyl amine (DIEA), and N-methylmorpholine (NMM) can be used at 2.5, 5, 7.5, or 10-fold molar excess as

10   in the standard peptide coupling method. The amino acid concentration can be used in 2.5, 5, 7.5, or 10-fold molar excess over the base concentration. Mixtures of CH_2Cl_2/DMF of 8:0, 7:1, 6:2, 5:3, 4:4 of 0:8, can be employed as solvents. In some cases, a linker which would "disappear" upon removal of the peptide from the substrate, can be used.

15   A free N-terminal amino group as is generated by cleavage of the immobilized peptides, using Glu or Gln linkers can provide an anchor for attaching peptides made with this strategy to glass slides or silicon wafers, which would allow very high throughput screening of libraries using microarray spotting and reading technology which has been developed for genomics studies.

20   The extent of coupling is currently qualitatively assessed by testing for free carboxyl groups using Malachite Green (Attardi et al., Tet. Lett., 41, 7391 (2000)), analogous to the use of ninhydrin to test for free amines. In some cases, sequence data from peptides might be useful and effective approaches for C-terminally sequencing peptides (succinylated peptides in solution and solid

25   phase) are now available (Samyn et al., Anal. Biochem., 72, 1389 (2000)).

These different resins have been tested – hydroxymethyl polystyrene and Pam resin (4-hydroxymethylphenylacetamidomethyl polystyrene) and MBHA. To provide the initial carboxyl group, a succinyl linker was used in preliminary studies. Some loss of polypeptide was observed during TFA (trifluoroacetic

30   acid) deprotection of t-butyl esters on hydroxymethyl polystyrene resin, but not significantly with Pam resin, and Pam resin was used in subsequent studies.

MBHA forms an amide linkage with the Glu linker that cleaves to an N-terminal glutamine (Glu) residue on the free peptide.

The initial coupling strategy used a five-fold excess of amine and coupling reagent. Excess coupling reagent and amine can be washed away after

5   coupling is complete. A number of such coupling reagents are known. Three have been tested to date: HATU, dicyclohexylcarbodiimide (DCC)/hydroxybenztriazol (HOBT) and O-Benzotriazol-1-yl-N,N,N',N'-tetramethyl uronium hexafluorophosphate (HBTU). The coupling methods have been tested by comparison of synthesis of the succinylated peptide Suc-Ala-Leu-

10   Pro-Phe.

Example 1

Preparation of Oligopeptide Using Succinylated Resin

Starting with commercial Pam resin, the resin was first succinylated

15   using succinic anhydride to provide a carboxyl group for subsequent coupling steps. Succinylation was selected for initial work since many protease related synthetic peptides are prepared with an N terminal succinyl group. Succinylated resin was then subjected to HBTU-mediated coupling of L-Ala-OtBu (L-Ala-t-butyl ester). Subsequently, the t-butyl ester was deprotected with 25%

20   TFA/DCM (1 × 5 seconds, filter, then 1 × 30 min). This process was repeated with each subsequent amino acid t-butyl ester (Ala, Pro, Phe to give Suc-Ala-Leu-Pro-Phe). Finally, the peptide was liberated from the polymer using TFMSA (trifluoromethylsulfonic acid)/TFA cleavage. The final product (Figure 2) was the desired product, and its identity was confirmed by HPLC-MS

25   comparison with commercially available authentic Suc-Ala-Leu-Pro-Phe.

Example 2

Comparison of Coupling Agents and Linkers

Using the Marfey's reagent based method cited above, HBTU and HATU

30   coupling procedures have been compared for synthesis of the test peptide Suc-Ala-Leu-Pro-Phe as summarized in Table 2.

Table 2: % D-isomer found in peptide Suc-Ala-Leu-Pro-Phe

	Ala	Leu	Pro	Phe
HBTU-coupling	27%	23%	<2%	<2%
HATU-coupling	38%	5%	<2%	<2%

5

With both coupling procedures >20% racemization of the first residue
attached to a succinyl linker is observed. HATU provides more effective
coupling in general than HBTU or DCC, and HATU shows ≤5% racemization of
subsequent residues even for the more difficult proline coupling. The
10   observation of high racemization of only the first residue following the succinyl
linker suggests that a protected α-amino group in a linker might reduce
racemization of the first added residue.

Z-Glu-OtBu was therefore tested as a linker. Z-Glu-OtBu was coupled to
PAM resin using the standard HATU-based coupling protocol, followed by the
15   standard deprotection and coupling procedure. This modification resulted in
<5% racemization of the residues in synthesized peptides. Peptides synthesized
with this linker strategy have a Glu residue as the N-terminus (Glu linker
strategy). To avoid the possibility of esterolytic cleavage of the resin-Glu
attachment, we have also tested the use of an amide attachment strategy by
20   coupling Z-Glu-OtBu to MBHA resin, a resin generally used in normal direction
peptide synthesis to general C-terminally amidated peptides. Cleavage from this
resin provides an N terminal Gln residue (i.e., amidated Glu side chain) (Gln
linker strategy). This linker strategy also provided peptides with low (<5%)
racemization in test syntheses. The increased stability of an amide link to the
25   resin may prove useful for some carboxyl group modification methods. One
advantage of both the Glu and Gln linker strategies over the succinyl linker
strategy is that they provide the free N-terminal α-amino group of the terminal
Glu or Gln residue which could be used to attach a multiplicity of peptides to
derivatized substrates such as to silicon wafers or glass slides comprising free
30   CHO groups, to yield "chip" libraries useful for very high throughput screening
(cf., MacBeath and Schreiber, Science, 289, 1760-176 (2000)).

12

Example 3

Inverse Peptide Synthesis Using MBHA Resin

MBHA-Z-Glu-OtBu resin (linked between the γ-carboxyl of Z-Glu-OtBu and the amine of MBHA resin) was employed. MBHA is a 4-methyl-benzhydryl

5 amine polystyrene resin. The coupling method used is the HATU/TMP-mediated activation. Details of this method are described below.

1.   Attachment of the Linker (Z-Glu-OtBu)

MBHA resin (1.0 equiv.) is first washed with DMF, 20% DIEA/DMF, DMF and DCM. To this pre-washed resin, a solution of Z-Glu-OtBu (5.0

10 equiv.) wherein Z is benzyloxycarbonyl, HATU (5.0 equiv.) and TMP (5.0 equiv.) in DMF is added. The suspension is stirred at room temperature for 3 h. The resin is then filtered and washed with DMF and DCM. the resulting resin is treated with a solution of acetic anhydride (3.0 equiv.) and DIEA (3.0 equiv.) in DMF for 30 min (to cap any remaining on-resin amine groups). The resin is

15 filtered and washed with DMF and DCM.

2.   Deprotection of tBu group

Above resin is treated with 25% TFA in DCM (1 × 5 s) and 50% TFA in DCM (30 min × 1). The resin then is washed with DCM × 3, NMP × 2, DCM × 3, dried.

20 3.   On-resin assembly of peptide-chain

The Z-Glu-linked resin is treated with a solution of an amino acid OtBu ester (5.0 equiv. usually HCl salt), HATU (5.0 equiv.) and TMP (10.0 equiv., 5 equiv. if amino acid is free base and not HCl salt) in DMF. The suspension is stirred at room temperature for 2 h. The resin is then filtered and washed with

25 DMF and DCM. A small amount of resin sample (1~5 mg) is removed and subjected to a color test reaction using 1 ml of 0.25% Malachite Green (in ethanol) along with 1 drop of triethylamine. A double coupling is performed if the testing shows positive (green or blue on resin). If the testing gives negative result (no color on resin), then repeat step 2 and step 3 until reaching the desired

30 length of peptide.

4.   Cleavage of peptide from the resin

        Before cleavage, the C-terminal protecting group (t-Bu) needs to be
removed using the identical procedure described in step 2. After being washed
and dried, the deprotected peptide-resin is treated with a mixture of TFA (100 µl)
5   and TFMSA (10 µl) for about 1h. The solution (crude product) is collected by
filtration and is ready for HPLC and LC/MS analysis.

Example 4

Introduction of common inhibitor functional groups, such as peptide aldehyde,
10   chloromethyl ketone, fluoromethyl ketone, and boronic acid, onto the
                C terminus of solid phase attached N-to-C peptide chains

        In preliminary studies, solution phase methods have been used to
synthesize a representative example of each of these inhibitor classes. For solid
phase syntheses, two distinct strategies will be required for this set of four
15   inhibitor classes.

        In the case of boronic acids and trifluoromethylketones, suitably
derivatized inhibitor monomers must be prepared and introduced into the peptide
chain. In the case of chloromethylketones and aldehydes, direct chemical
modification of the C terminus can provide the desired functional group.
20   Alanine is the naturally preferable P1 residue for the PBPs, and an acceptable
residue for HLE.

A.   Boronic acid (boro-Ala) based peptides (-X-Y-Z-boroAla)

        The chemistry required to prepare boroAla and several other hydrophobic
amino acid analogs such as boroVal is well known (Kettner & Shenvi, J. Biol.
25   Chem., 259, 15106 (1984); Matteson & Sadhu, U.S. Pat. No. 4,525,309 (1985)).
See Fig. 3. Boro-Ala can be introduced onto the C-terminus of a peptide using
the standard procedures for adding an amino acid to a chain.

B.   Peptide aldehyde based peptides (-X-Y-Z-CHO)

        As shown in Fig. 4, several strategies for the synthesis of peptide
30   aldehydes have been described, including from amino alcohols by oxidation
(Thompson, Biochem., 12, 47 (1973); Okura & Swern, Tetrahedron, 34, 1651

14

(1978)), by reduction of the amino acid methyl esters with diisobutylaluminum hydride (DIBAL) (Ito et al., Biochem. Biophys. Res. Comm., 49, 343 (1975); Gorenstein & Shaw, Biochem., 21, 4679 (1982)), or by reduction of Weinreb N-methoxy-N-methylcarboxamides with LiAlH$_4$ (Fehrentz & Castro, Synthesis,

5   676 (1983)). The best solution phase method tested so far is reduction of Weinreb amides. This method works well in the presence of Boc and Cbz groups, but may be incompatible with most esters.

There are a number of reducing agents of varying strength (Hudlicky, Reagents In Org. Chem., ACS, (2d ed. 1998)) which can be applied to this

10   problem. Several reducing agents of different power can be tested against a series of esters. Of particular interest is the possibility of generating active HOBT or HOAT esters, as is generated in the standard coupling reaction, and reducing them with a relatively mild reducing agent such as LiAlH(OtBu)$_3$. The possible coordinating effect of the active ester could facilitate such a reaction

15   and assist in stopping the reaction at the aldehyde stage as occurs in reduction of Weinreb amides with LiAlH$_4$, possibly without reduction of side chain protected esters. Such a strategy should allow solid phase peptide aldehyde preparation via a simple modification of the standard reverse peptide synthesis strategy.

The successful Pfitzner-Moffat oxidation (DCC/Cl$_2$HCO$_2$H/DMSO) of

20   the peptide trifluoroamino alcohol suggests this strategy could also work for synthesis of peptide aldehydes as outlined in Fig. 4. This approach was tested, and works well in generating an on resin aldehyde, but substantial degradation of the peptide aldehyde occurs during TFMSA/TFA cleavage from the resin. Protection of the aldehyde by treatment with a 20-fold excess of ethylene glycol

25   in 5% TFA/DCM followed by cleavage from the resin provided the protected aldehyde (Gln-Phe-Lys-Ala-CH(OCH$_2$)$_2$ as the major peptide product ([M+H] = 522.8). Impurities were: a) Gln-Phe-Lys ([M+H]: 421.5) due to incomplete coupling of the amino alcohol, b) Gln-Phe-Lys-Ala-CH$_2$OH([M+H]: 486.6), due to incomplete oxidation of the amino alcohol, and c) an unknown impurity

30   ([M+H]: 574.8). Reaction of on resin peptide aldehyde with nitroethane and dithioethane have also been tested and give complete conversion of the aldehyde

to the expected derivatives (Fig. 5). In Fig. 5, Z= benzyloxycarbonyl, • = resin, and Z-Glu-OtBu is PhCH$_2$OC(=O)NHCH(CH$_2$CH$_2$CO$_2$H)-CO$_2$tBu.

A large number of commercially available nucleophiles (cf. nitroethane) can be reacted with on-resin aldehydes to provide peptide aldehyde adduct
5   libraries of tremendous diversity. Dithiane and other derivatives provide the further possibility of acyl-anion equivalent chemistry, which would allow peptide aldehydes to be further elaborated using various alkylating agents into peptide mimetic ketones (see, Seebach et al., J. Org. Chem. 40, 231 (1975); Hase et al., Aldrichimica Acta, 14, 73 (1981).

10 C.   Trifluoromethylketone-based peptides (X-Y-Z-Ala-CF$_3$)

The classic procedure for preparing peptide trifluoromethylketones is shown in Fig. 6 (Imperiali & Abeles, Tet. Lett., 22, 135 (1986)). Oxidation of the acyl trifluoromethyl aminoalcohol was performed with KMnO$_4$, but can also be performed with a modified Pfitzner-Moffat (carbodiimide/Cl$_2$HCO$_2$H/DMSO)
15 procedure (Fearon et al., J. Med. Chem., 30, 1617 (1987)), or with a Dess-Martin periodinane (1,1,1-triacetoxy-2,1-benzoxiodol-3-(3H)-one) procedure (Edwards et al., US. Pat. No. 5,194,588 (1993); Dess & Martin, J. Org. Chem., 48, 4155 (1983)). On-resin trifluoromethyl ketones have been prepared using the modified Pfitzner-Moffat procedure described above.

20   An alternative strategy is to protect the amino group of the aminotrifluoromethyl alcohol with Boc$_2$O, oxidize the Boc-aminotrifluoromethyl alcohol to the ketone, followed by removal of the Boc group with HBr/HOAc to provide the unprotected trifluoromethyl amino ketone as the HBr salt. In the event an on resin oxidation approach is used, methionine and tryptophane-
25 containing peptides might also be oxidized, but would not be oxidized if the trifluoromethyl amino ketone monomer approach is successful.

There are additional approaches for preparing perfluoroalkyl ketones which involve the addition of metallo perfluoralkyl anions to suitable amino acid derivatives, such as addition of trifluoromethyl zinc to amino acid aldehydes and
30 addition of pentafluoroethyl lithium to protected amino acid esters.

D.   Chloromethyl ketone-based libraries (-X-Y-Z-CH₂-Cl)

Peptide chloromethylketones are traditionally synthesized by coupling a free carboxylic acid to diazomethane using an isobutylchloroformate mixed anhydride procedure, followed by treatment with HCl (Fig. 7) (Schoellmann &

5   Shaw, Biochem., 2, 252 (1963); Green & Shaw, J. Biol. Chem., 256, 1923 (1981)).

Although chloromethyl ketones are unlikely to have clinical application, given their alkylating capability, they form covalent adducts with their targets and have a number of biochemical applications in the study of structure/function

10   relationships in proteins. Chloromethyl ketones are also excellent synthetic intermediates elaborated into a number of interesting protease inhibitor classes, such as hydroxyethylene inhibitors which are effective inhibitors of HIV protease (cf. Dreyer et al., Biochem., 31, 6646 (1992); Konvalinka et al., Eur. J. Biochem., 250, 559 (1997)), and the Alzheimer's disease-related β-secretase

15   enzyme (Shearman et al., Biochem., 39, 8698 (2000), among others.

The proposed method for inverse peptide synthesis has been further refined and can now be used to synthesize peptides with acceptably low levels of racemization. A method for generating inverse peptide trifluoromethyl ketones has been demonstrated and this method is appropriate for library synthesis. One

20   possible route to inverse peptide aldehydes has been tested and found to provide on-resin aldehydes. Resin aldehydes can be protected and eluted from the resin. The method of inverse peptide synthesis is capable of providing a wide variety of C-terminally modified peptide mimetics.

All cited publications, patent applications, and patents are incorporated

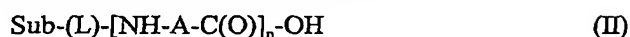25   by reference herein as though fully set forth.

17

What is claimed is:

1.   A method for preparing a peptide of formula (I):
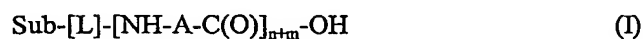
$$\text{Sub-[L]-[NH-A-C(O)]}_{n+m}\text{-OH} \qquad \text{(I)}$$

5   comprising:

(a)   reacting an immobilized compound of formula (II):

$$\text{Sub-(L)-[NH-A-C(O)]}_{n}\text{-OH} \qquad \text{(II)}$$

with an amino acid ester or peptide derivative of formula (III):

$$\text{H-[NH-A-C(O)]}_{m}\text{-O(tBu)} \qquad \text{(III)}$$

10   in the presence of a coupling agent to yield a peptide compound

of general formula (IV):

$$\text{Sub-[L]-[NH-A-C(O)]}_{n+m}\text{-O(tBu)} \qquad \text{(IV)};$$

(b)   removing the tBu (t-butyl) group to produce a solid-support

bound carboxylic acid or peptide derivative of general formula

15   (I);

wherein n is a positive integer, m is a positive integer, Sub is a solid support, L is

a linker to the amino acid ester or peptide derivative, each A independently is the

residue of an amino acid; a peptide residue; or the structure -NH-A represents a

heterocyclic group.

20

2.   The method of claim 1 which further comprises, prior to step (a):
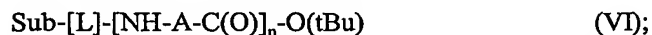
forming a solid-support bound compound of general formula (VI):

$$\text{Sub-[L]-[NH-A-C(O)]}_{n}\text{-O(tBu)} \qquad \text{(VI)};$$

by reacting a functionalized solid support of general formula Sub[L]X

25   with a compound of formula: $\text{H-[NH-A-C(O)]}_{n}\text{-OH}$, wherein X is a group that

links L to NH_2-A by elimination of XH; and

removing the t-Bu group to yield a compound of formula (II).

3.   The method of claim 1 or 2 comprising cleaving compound (IV) at the L-

30   NH bond to yield a compound of formula (IX);

$$\text{H-[HN-A-C(O)]}_{n+m}\text{-OH.}$$
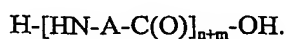
4.   The method of claim 1 further comprising, following step (b); carrying

out, x times, the steps of reacting compound (I) with a compound of formula (III)

and removing the tBu group to yield a compound of formula (VIII):

$$\text{Sub-(L)-[NH-A-C(O)]}_{n+m(x+1)}\text{-OH} \qquad\qquad \text{(VIII)}$$
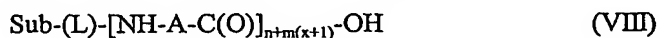
5   wherein x is an integer.

5.   The method of claim 2 wherein [L]X is $-OC(O)(CH_2)_2CH(NHZ)CO_2H$,

wherein Z is a protecting group.

10   6.   The method of claim 2 wherein [L]X is $HNC(O)(CH_2)_2CH(NHZ)CO_2H$,

wherein Z is a protecting group.

7.   The method of claim 5 or 6 wherein Z is benzyloxycarbonyl (Cbz).

15   8.   The method of claim 2 wherein [L]X is $-C(O)(CH_2)_2CO_2H$.

9.   The method of claim 3 wherein each A is individually the residue of an

α-amino acid.

20   10.   The method of claim 3 wherein each A is individually the residue of a

naturally occurring L-amino acid.

11.   The method of claim 1 wherein the coupling agent is HATU.


25   12.   The method of claim 1 or 2 wherein the tBu group is removed with TFA.

13.   The method of claim 3 wherein the cleavage is carried out using

TFMSA/TFA.

30   14.   The method of claim 3 wherein Sub is a polymer comprising free NH_2 or

OH groups.

15.   The method of claim 14 wherein the polymer comprises polystyrene.

16.   The method of claim 1 comprising reacting compound (I) with H_2N(alkyl)CH(OH)CF$_3$ (XV) to yield the corresponding amide of formula (I) and

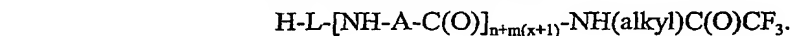5   oxidizing the CH(OH) moiety to yield a compound of general formula (XII):

$$\text{Sub-(L)-[NH-A-C(O)]}_{n+m}\text{-NH(alkyl)C(O)CF}_3 \qquad \text{(XII).}$$
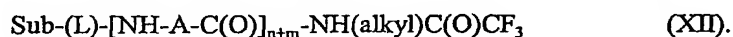
17.   The method of claim 16 further comprising cleaving compound (XII) from Sub to yield a compound of formula:

10 $$\text{H-L-[NH-A-C(O)]}_{n+m}\text{-NH(alkyl)C(O)CF}_3.$$
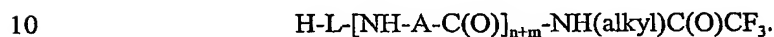
18.   The method of claim 4 comprising reacting compound (VIII) with compound (XV) to yield the corresponding amide of formula (VIII) and oxidizing the CH(OH) moiety to yield a compound of general formula (XVI):

15   $$\text{Sub-(L)-[NH-A-C(O)]}_{n+m(x+1)}\text{-NH(alkyl)C(O)CF}_3 \qquad \text{(XVI).}$$
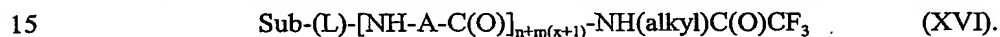
19.   The method of claim 17 further comprising cleaving compound (XVI) from Sub to yield a compound of formula:

$$\text{H-L-[NH-A-C(O)]}_{n+m(x+1)}\text{-NH(alkyl)C(O)CF}_3.$$

20

20.   The method of claim 1 further comprising reacting compound (I) with H_2N(alkyl)OH (XVII) to yield the hydroxyalkylamide of (I) and oxidizing the terminal hydroxy group to yield a compound of formula (XIII):

$$\text{Sub-(L)-[NH-A-C(O)]}_{n+m}\text{-NH(alkyl)CHO} \qquad \text{(XIII).}$$

25
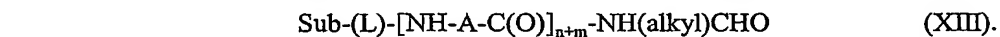
21.   The method of claim 4 further comprising reacting compound (VIII) with H_2N(alkyl)OH (XVII) to yield the hydroxyalkylamide of (VIII) and oxidizing the terminal hydroxy group to yield a compound of formula (XVIII):

$$\text{Sub-(L)-[NH-A-C(O)]}_{n+m(x+1)}\text{-NH(alkyl)CHO} \qquad \text{(XVIII).}$$
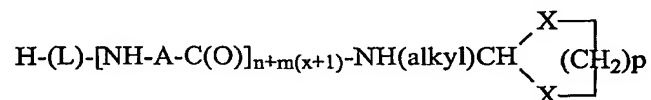
30
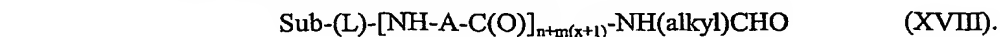
22.   The method of claim 20 further comprising protecting the terminal CHO group of compound (XIII).

23.   The method of claim 21 further comprising protecting the terminal CHO
5   group of compound (XVIII).

24.   The method of claim 22 or 23 wherein the CHO group is converted into an ethylene glycol acetal, a propylene glycol acetal, an ethylene dithiol acetal or a 1,3-dithiane.
10

25.   The method of claim 24 further comprising cleaving protected compound from the substrate to yield a compound of formula:

$$H\text{-}(L)\text{-}[NH\text{-}A\text{-}C(O)]_{n+m(x+1)}\text{-}NH(alkyl)CH \overset{X}{\underset{X}{\diagup}} \overset{\bigsqcup}{\underset{\bigsqcup}{(CH_2)p}}$$

wherein X is 0 or S, and p is 0 or 1.

15   26.   The method of claim 4 further comprising reacting compound (VIII) with a boroamine to yield a compound of formula (XIV):

$$Sub\text{-}[L]\text{-}[NH\text{-}A\text{-}C(O)]_{n+m(x+1)}\text{-}NH(A)B(OR)_2 \qquad (XIV),$$
wherein R is $(C_1\text{-}C_6)$alkyl, phenyl, or the residue of a diol.

20   27.   The method of claim 1 further comprising reacting compound (I) with a boroamine to yield a compound of formula (XIX):
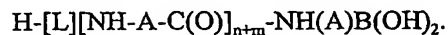
$$Sub\text{-}[L]\text{-}[NH\text{-}A\text{-}C(O)]_{n+m}\text{-}NH(A)B(OR)_2 \qquad (XIX),$$
wherein R is $(C_1\text{-}C_6)$alkyl, phenyl, or the residue of a diol.
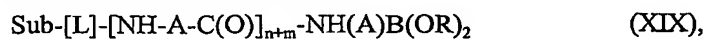
25   28.   The method of claim 26 further comprising cleaving compound (XIV) to yield a compound of the formula:

$$H\text{-}[L]\text{-}[NH\text{-}A\text{-}C(O)]_{n+m(x+1)}\text{-}NH(A)B(OH)_2.$$

29.   The method of claim 27 further comprising cleaving compound (XIX) to yield a compound of formula:

$$H-[L][NH-A-C(O)]_{n+m}-NH(A)B(OH)_2.$$

5   30.   The method of claim 1 or 2 wherein n is 1.

31.   The method of claim 1 or 2 wherein m is 1.

32.   The method of claim 30 wherein m is 1.

10

33.   The method of claim 4 wherein x is 1-10.

34.   The method of claim 32 wherein x is 1-10.

15   35.   The method of claim 33 wherein x is 1-5.

36.   The method of claim 34 wherein x is 1-5.

37.   A compound of formula (I), (II), (IV), (VI) or (VIII).

20

38.   A compound of formula $Sub-(L)-[NH-A-CO]_{n+m(x+1)}-O(tBu)$.

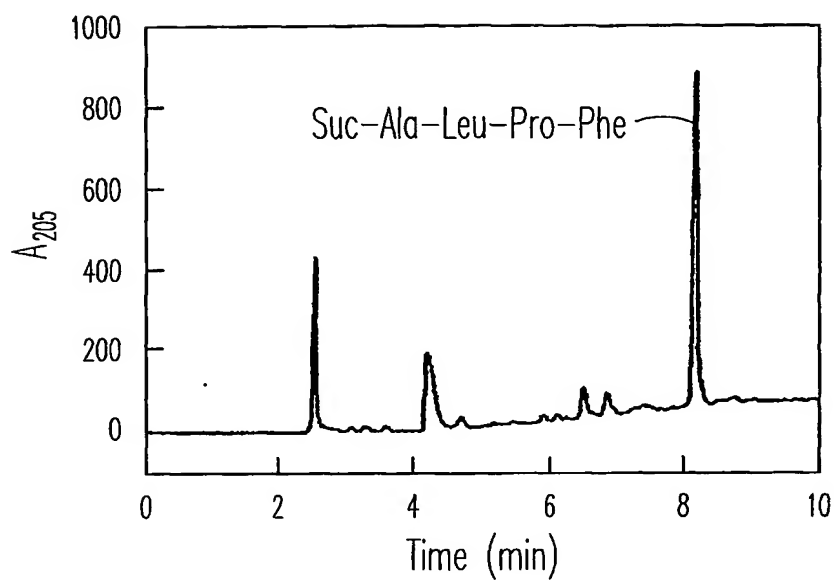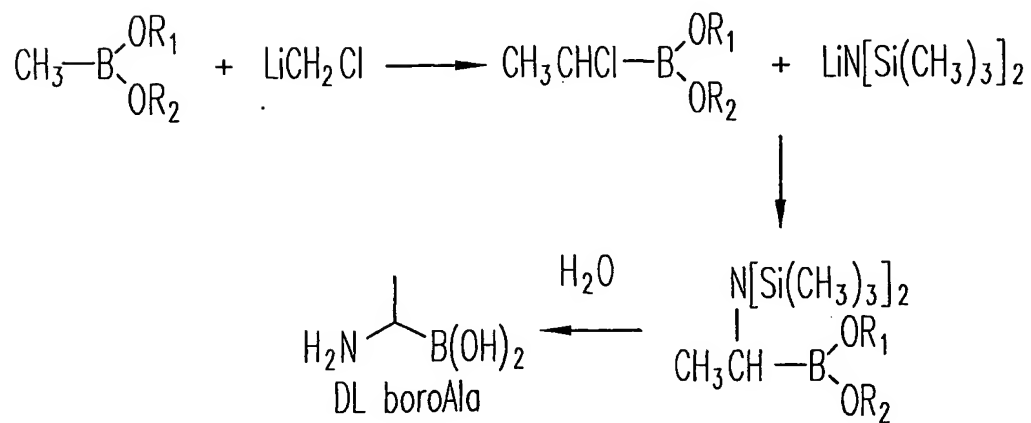39.   A compound of claim 37 or 38 wherein n = 1.

25   40.   A compound of claim 39 wherein m = 1.
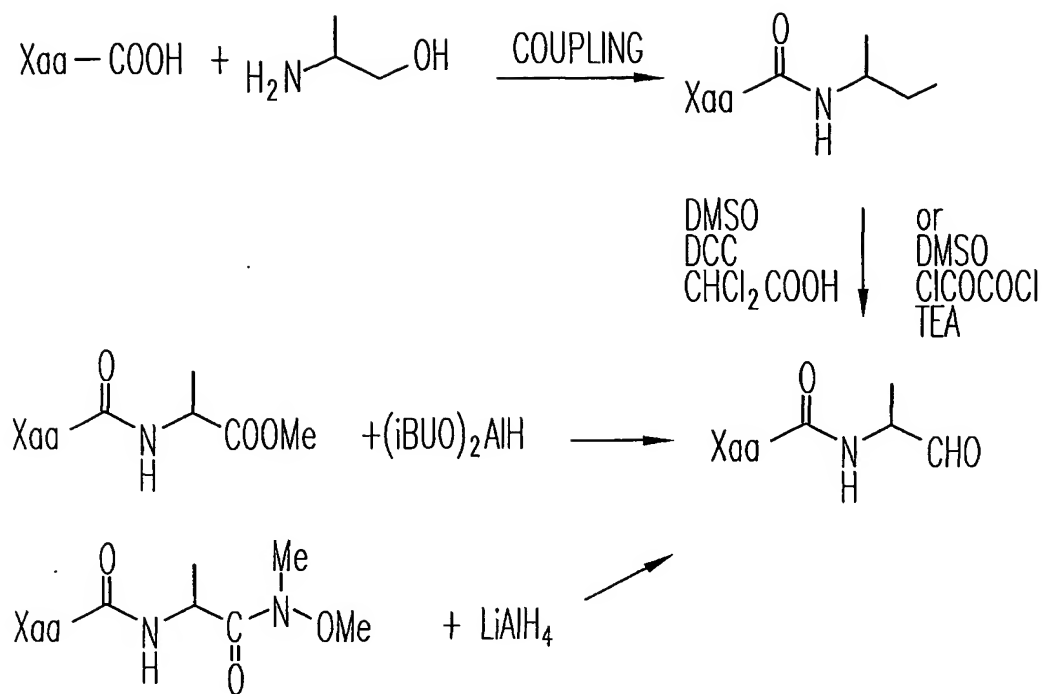
41.   A compound of claim 40 wherein x = 1.
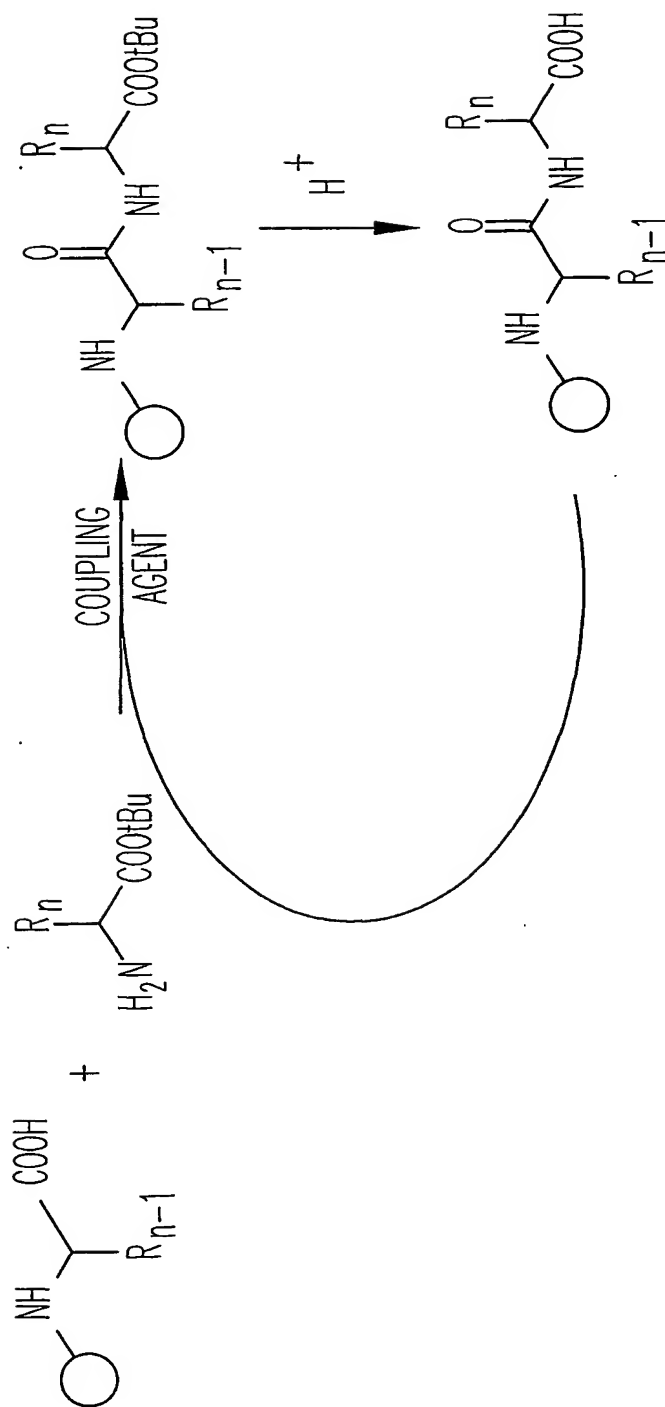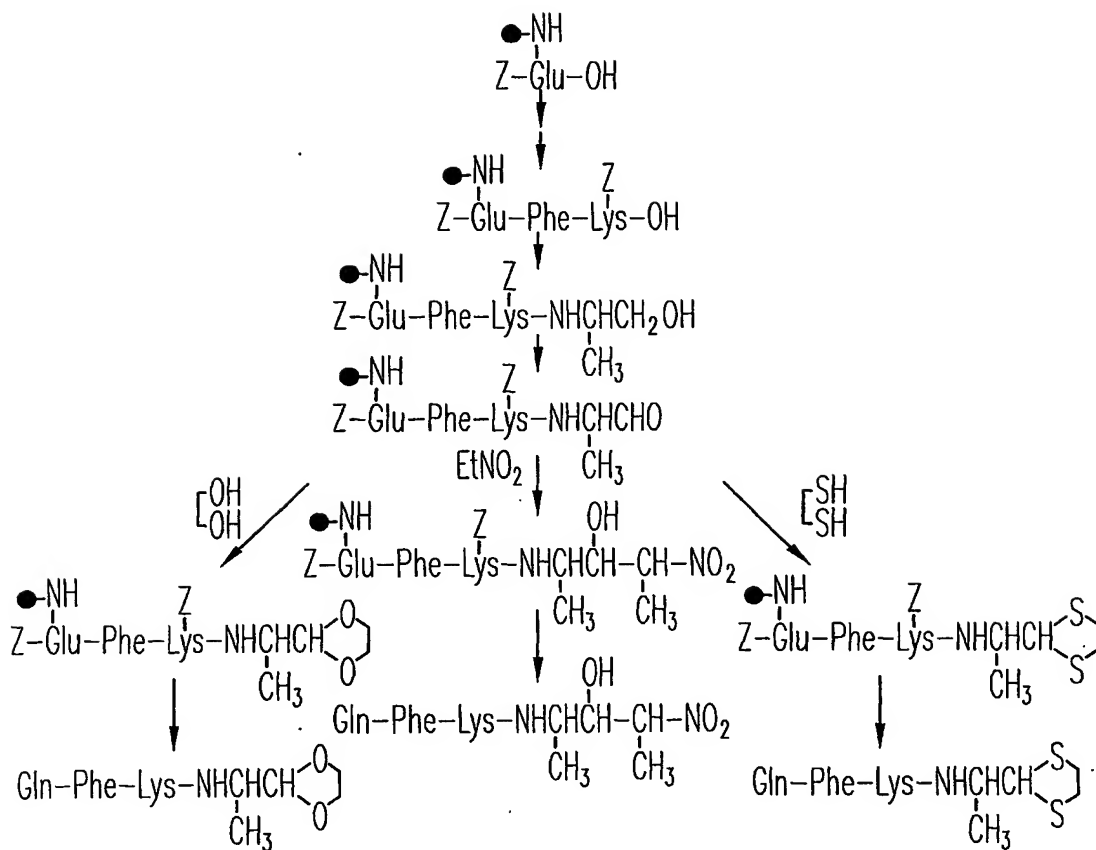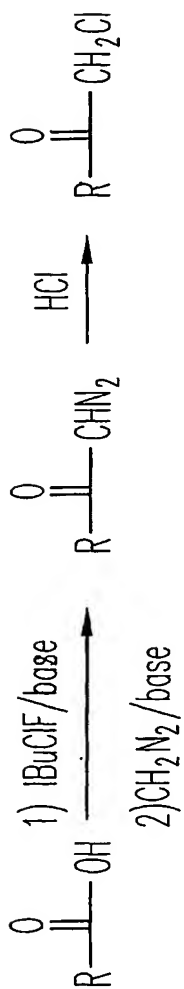
22

Fig.1

Fig.2

Fig.3

Fig. 4

Fig. 5

Fig.6
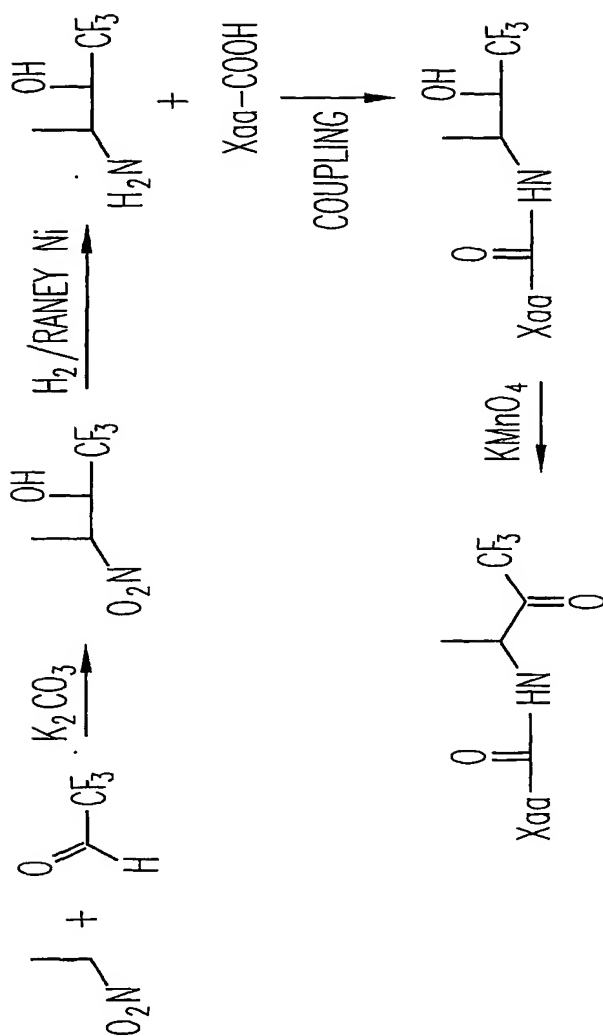
Fig. 7

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C07K 1/04
US CL : 530/334

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/334

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, CAS Online

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A --- X	WO 90/005738 A (PUBLIC HEALTH LABORATORY SERVICE BOARD) 31 May 1990, see entire document, especially pages 11-12.	1-36, 38-41 ----------- 37
A	WO 93/005065 A1 (PORTION DEVELOPMENTS LIMITED) 18 March 1993, see entire document.	1-41

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

*	Special categories of cited documents:
"A"	document defining the general state of the art which is not considered to be of particular relevance
"E"	earlier document published on or after the international filing date
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O"	document referring to an oral disclosure, use, exhibition or other means
"P"	document published prior to the international filing date but later than the priority date claimed

"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 AUGUST 2002	2 5 SEP 2002

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	DAVID LUKTON
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)*